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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
C12N 15/12, C07K 14/705, C12N 5/10, C12Q 1/68, G01N 33/68

A1

(11) International Publication Number: WO 00/63371

(43) International Publication Date: 26 October 2000 (26.10.00)

(21) International Application Number: PCT/GB00/01455

(22) International Filing Date: 17 April 2000 (17.04.00)

(30) Priority Data:
9908513.6 15 April 1999 (15.04.99) GB

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(81) Designated States: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: PROTEASE ACTIVATED RECEPTOR 2 VARIANTS

(57) Abstract

A variant PAR–2 receptor polypeptide or a fragment thereof is provided which (i) has reduced sensitivity to trypsin as compared with wild type PAR–2; (ii) has increased sensitivity to trans–cinnamoyl–LIGRLO–NH_2 as compared with wild type PAR–2; and (iii) is activated by TLIGRL–NH_2, which polypeptide or fragment thereof comprises an extracellular loop 2 (ECL–2) having at least one amino acid difference from the corresponding ECL–2 amino acid sequence of the wild type polypeptide.

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PROTEASE ACTIVATED RECEPTOR 2 VARIANTS

Field of the invention

The present invention relates to the identification of polymorphic form of protease activated receptor 2 polypeptide having altered properties compared to wild type.

Background to the invention

The biological actions of the proteases thrombin, trypsin and tryptase are increasingly being attributed to the activation of a novel class of G-protein regulated receptors termed protease activated receptors (PARs) (1). Proteolytic cleavage of a PAR amino-terminal exodomain unmasks a tethered ligand that binds intramolecularly onto the body of the receptor to initiate signaling. Currently, four PARs have been cloned of which PAR-1 and PAR-3 are activated by thrombin (2,3), PAR-2 is activated by trypsin and mast cell tryptase (4-6), PAR-4 is activated by both thrombin and trypsin (7). With the exception of PAR-3, synthetic peptides corresponding to the first five amino acids of the receptor's tethered ligand can activate the PARs (1). SLIGKV-NH_2, is used to activate human PAR-2, although the mouse sequence SLIGRL-NH_2 and the selective PAR-2 agonist tc-LIGRLO-NH_2 are more potent activators of PAR-2 (8). The human PAR-1 agonist SFLLR-NH_2 has proved to be a less reliable agonist of PAR-1 because it activates human PAR-2 (9,10). However, the highly selective PAR-1 agonists, TFLLR-NH_2 and Ala-parafluoroPhe-Arg-cyclohexylAla-Citrulline-Tyr (Cit)-NH_2 are now more commonly used to selectively activate PAR-1. The human PAR-4 peptide agonist GYPGQV-NH_2, unlike the other peptides, requires higher concentrations to stimulate receptor activation (7). Consequently, these small peptides have proved to be useful tools for identifying the potential biological roles of PARs *in vivo*.

PAR-2 is expressed on a variety of cells including epithelial cells (2,11), endothelial cells (12,13), smooth muscle cells (14), keratinocytes (15,16), neutrophils (17) and some T-cell lines (18). Considerable evidence now implicates PAR-2 as an important receptor in inflammation. For example, the inflammatory mediators TNF, IL-1 and LPS upregulate endothelial PAR-2 expression (19), whilst PAR-2 activation results in inflammatory

cytokine release from keratinocytes (16) and the rolling and adhesion of leukocytes to the endothelium (20). Moreover, the administration of the PAR-2 agonist (SLIGRL-NH_2) *in vivo* induces an inflammatory response (21). The resulting edema occurs via neuronal PAR-2 activation (22). Mast cell tryptase, a major mediator of inflammation and activator of PAR-2, stimulates cytokine release from human endothelial (23) and epithelial cells (24), and when injected into the skin of guinea pigs induces vascular leakage (25) and the accumulation of eosinophils and neutrophils (26). Because the proteases trypsin and tryptase can initiate an inflammatory response by the selective activation of PAR-2, receptor polymorphisms that can alter receptor activation may have important implications for disease.

The exact mechanism whereby the tethered ligand activates PAR-2 remains to be clearly established. Elegant studies with the closely related thrombin receptor (PAR-1) have indicated the tethered ligand interacts with extracellular loop-2 (ECL-2) (27-29). In addition it appears that ECL-2 is important for governing agonist specificity and receptor signaling. Recent evidence has also implicated ECL-2 of PAR-2 as a critical region for agonist specificity (30). These studies demonstrated that the PAR-2 agonist peptides (PAR-2AP) SLIGRL-NH_2 (which is unable to activate PAR-1) could activate a PAR-1 chimera wherein ECL-2 of PAR-2 was substituted into PAR-1. In addition, mutating residues in ECL-2 of PAR-2 that were found to be of importance in PAR-1 significantly alter receptor function (31). Thus, it would appear that PAR-1 and PAR-2 use the same extracellular domains to initiate receptor activation.

Summary of the invention

In the course of cloning PAR-2 from a human colonic cDNA library, we have identified a polymorphic form of human PAR-2 (PAR_2F240S) characterized by a phenylalanine to serine mutation at residue 240 of extracellular loop-2 (ECL-2). Genomic analysis of multiple individuals confirmed the existence of both alleles, with allelic frequencies of 0.916 (F_{240}) and 0.084 (S_{240}) for the wild-type and mutant alleles respectively. Calcium signaling was used as an index of receptor activation in permanently transfected cell lines. PAR_2F240S displayed a significant reduction in sensitivity towards trypsin (~ 3.7-fold), the PAR-activating peptides, SKLIGV-NH_2 (~2.5-fold) and SLIGRL-NH_2 (~2.8 fold), but

an *increased* sensitivity towards the selective PAR-2 agonist, *trans-cinnamoyl*-LIGRLO-NH_2 (4-fold). Increased sensitivity was also observed towards the selective PAR-1 agonist, TFLLR-NH_2 (~7 fold), but not to other PAR-1 agonists tested. Although the PAR-4 agonist GYPGKF-NH_2 was without effect, trans-cinnamoyl-YPGKF-NH_2 could selectively activate the PAR_2F240S receptor but not the wild type receptor. Furthermore, we found that TLIGRL-NH_2 is a selective PAR_2F240S agonist. By introducing the F240S mutation into rat-PAR-2, we observed shifts in agonist potencies that mirrored those seen with the human PAR_2F240S confirming that F240 plays a key role in agonist specificity. The dramatically altered pharmacological profile induced by this polymorphism will have important implications for the design of PAR-targeted antagonists and may contribute to, or be predictive of an inflammatory disease.

Accordingly, the present invention provides a variant protease activated receptor 2 (PAR-2) polypeptide or a fragment thereof which

(i) has reduced sensitivity to trypsin as compared with wild type PAR-2;

(ii) has increased sensitivity to trans-cinnamoyl-LIGRLO-NH_2 as compared with wild type PAR-2; and

(iii) is activated by TLIGRL-NH_2,

which polypeptide or fragment thereof comprises an extracellular loop 2 (ECL-2) having at least one amino acid difference from the corresponding ECL-2 amino acid sequence of the wild type polypeptide.

Preferably the wild type polypeptide used for comparison purposes is human PAR-2 having the amino acid sequence set out in SEQ ID No.2.

Preferably the at least one amino acid difference includes a modification at residue 240 of the amino acid sequence set out in SEQ ID No.2, or its equivalent, more preferably a substitution at residue 240 of the amino acid sequence set out in SEQ ID No. 2, or its equivalent. Thus in a preferred embodiment, the present invention provides a PAR-2 polypeptide or fragment thereof which comprises a substitution at residue 240, or its equivalent, such that residue 240 is not phenylalanine. Preferably amino acid residue 240 is not an aromatic amino acid.

Preferably, a polypeptide of the invention comprises a phenylalanine to serine substitution at residue 240 of the amino acid sequence set out in SEQ ID No.2, or its equivalent.

The present invention also provides a nucleotide encoding a polypeptide of the invention and a host cell comprising a nucleotide of the invention operably linked to a transcriptional control sequence capable of directing the expression of a polypeptide of the invention in the host cell. Preferably a nucleotide of the invention comprises a thymidine residue at position 719 of the sense strand of the coding region of the nucleotide sequence set out in SEQ ID No. 1.

In a further aspect, the present invention provides a pair of oligonucleotide primers, each comprising at least 10 contiguous nucleotides of the polynucleotide of the invention, which primers are suitable for use in a polymerase chain reaction for amplifying the ECL-2 region of a PAR-2 gene. The present invention further provides the use of said primers in a method of identifying an individual having a polymorphism in the ECL-2 region of one or both PAR-2 gene alleles.

In another aspect, the present invention provides a method of identifying a compound that modulates the activity of a polypeptide of the invention which method comprises contacting said polypeptide with said a candidate compound and determining whether the candidate compound modulates the activity of said polypeptide.

In a further embodiment, the present invention provides a method of identifying a compound that selectively inhibits a polypeptide of the invention which method comprises:

(i) contacting said polypeptide with a candidate compound in the presence and absence of a PAR-2 agonist;

(ii) selecting those candidate compounds that inhibit or reduce the agonist-mediated activation of the polypeptide;

(iii) contacting a wild type PAR-2 polypeptide with a candidate compound selected in step (ii) in the presence and absence of a PAR-2 agonist; and

(iv) selecting those candidate compounds that do not significantly inhibit the agonist-mediated activation of the wild type PAR-2 polypeptide.

The present invention further provides a compound identified by the above methods of the invention and its use in treating a patient comprising a polypeptide of the invention. Thus the present invention provides a compound that modulates the activity of a polypeptide of the invention for use in a method of treating a condition characterized by inflammation. Preferably, the compound inhibits the activity of a polypeptide of the invention.

In another aspect, the present invention provides a method of identifying a patient having an increased risk of inflammatory disorders which method comprises determining the presence or absence of a polypeptide of the invention or a polynucleotide of the invention in a biological sample obtained from said patient.

Detailed description of the invention

Although in general the techniques mentioned herein are well known in the art, reference may be made in particular to Sambrook *et al.*, Molecular Cloning, A Laboratory Manual (1989) and Ausubel *et al.*, Short Protocols in Molecular Biology (1999) 4th Ed, John Wiley & Sons, Inc.

A. PAR-2 variant polypeptides

The sequence of wild type PAR-2 has been published for human, mouse and rat PAR-2 (5, 37, 36, respectively). These sequences are available in public databases under Genbank accession numbers P55085 and Z49994 (human), P55086 (mouse) and Q63645 (rat). Other PAR-2 sequences may be obtained from other species such as other primate species and rodent species. Such PAR-2 sequences will generally be at least 60, 70 or 80% identical at the amino acid level over at least 50 or 100 amino acids with the human sequence shown as SEQ ID NO 2. In particular, homology should typically be considered with respect to those regions of the sequence known to be essential for PAR-2 function rather than non-essential neighbouring sequences. Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of the present invention it is preferred to express homology in terms of sequence identity.

Homology comparisons can be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs can calculate % homology between two or more sequences.

% homology may be calculated over contiguous sequences, i.e. one sequence is aligned with the other sequence and each amino acid in one sequence directly compared with the corresponding amino acid in the other sequence, one residue at a time. This is called an "ungapped" alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues (for example less than 50 contiguous amino acids).

Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion will cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in % homology when a global alignment is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without penalising unduly the overall homology score. This is achieved by inserting "gaps" in the sequence alignment to try to maximise local homology.

However, these more complex methods assign "gap penalties" to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - will achieve a higher score than one with many gaps. "Affine gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties will of course produce optimised alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example when using the GCG Wisconsin Bestfit package (see below) the default gap penalty for amino acid sequences is -12 for a gap and -4 for each extension.

Calculation of maximum % homology therefore firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (University of Wisconsin, U.S.A.; Devereux *et al.*, 1984, Nucleic Acids Research 12:387). Examples of other software than can perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel *et al.*, 1999 *ibid* – Chapter 18), FASTA (Atschul *et al.*, 1990, J. Mol. Biol., 403-410) and the GENEWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online searching (see Ausubel *et al.*, 1999 *ibid*, pages 7-58 to 7-60). However it is preferred to use the GCG Bestfit program.

Although the final % homology can be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public default values or a custom symbol comparison table if supplied (see user manual for further details). It is preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62.

Once the software has produced an optimal alignment, it is possible to calculate % homology, preferably % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

PAR-2 sequences other than the human, mouse and rat sequences referred to above can, for example, be obtained using standard cloning techniques or by searching public databases.

The present invention provides variant PAR-2 polypeptides of the invention which differ from the wild type sequences in that the extracellular loop 2 (ECL-2) has at least one amino acid difference from the corresponding ECL-2 amino acid sequence of the wild type polypeptide. The amino acid change is such that the variant PAR-2 polypeptide has the following properties:

(i) it has reduced sensitivity to trypsin as compared with wild type PAR-2:

(ii) it has increased sensitivity to trans-cinnamoyl-LIGRLO-NH_2 as compared with wild type PAR-2; and

(iii) is activated by TLIGRL-NH_2,

The extracellular loop 2 of human PAR-2 is located at approximately residues 212 to 245 of the sequence shown as SEQ ID No. 2, or an equivalent region in PAR-2 sequences from other species. Thus, a variant PAR-2 polypeptide of the invention will have at least one amino acid difference in this region from the wild type sequence. The comparison should be made with the corresponding wild type sequence. The term "wild type" is known in the art and is generally taken to mean the phenotype that is characteristic of most of the members of occurring naturally and contrasting with the phenotype of a mutant. Preferably, in the present context, the wild type sequence is taken to be the human wild type amino acid sequence shown as SEQ ID NO 2.

In a preferred embodiment, the amino acid difference is located between (and including) residues 236 to 245 of the sequence shown as SEQ ID NO. 2 (all further references to particular amino acid numbering should be taken to include the corresponding/equivalent residues in other PAR-2 sequences - the term "or its equivalent" refers to the amino acid in other PAR-2 sequences that corresponds to a particular numbered residue of the human sequence set out in SEQ ID. No. 2). More preferably, the difference is a deletion, substitution or insertion, preferably a substitution, at residue 240. In particular, a variant PAR-2 polypeptide of the invention comprises a non-aromatic amino acid at residue 240, preferably serine.

In another embodiment, a variant PAR-2 polypeptide of the invention does not comprise an aromatic residue , such as phenylalanine, at residue 240, or its equivalent in other PAR-2 sequences.

Variant polypeptides of the invention can be tested to determine their sensitivity to trypsin and trans-cinnamoyl-LIGRLO-NH_2 using, for example, the assays described in the Examples. Thus, typically, trypsin sensitivity is measured by exposing cells that express the variant PAR-2 polypeptide to trypsin and measuring the calcium response (see

reference 31 for details of the calcium cell-signaling assay). Sensitivity to the selective PAR-2 agonist trans-cinnamoyl-LIGRLO-NH_2 can also be determined using a similar assay. A comparison is then typically made with wild type PAR-2 tested under similar conditions.

Preferably, a polypeptide of the invention is at least two-fold less sensitive to trypsin than is wild type PAR-2, more preferably at least three-fold less sensitive. Further, a polypeptide of the invention is preferably at least two-fold more sensitive to trans-cinnamoyl-LIGRLO-NH_2 than is wild type PAR-2, more preferably at least three or four-fold more sensitive. In addition a polypeptide of the invention is preferably no more than 20 or 40-fold more sensitive to trans-cinnamoyl-LIGRLO-NH_2 than wild type PAR-2.

Variant polypeptides of the invention are also preferably activated by TLIGRL-NH_2. Activation can again, typically be tested using a calcium cell-signaling assay, such as the assay described in reference 31.

Polypeptides of the invention also include fragments of the above variant full length sequences provided that they comprise at least the region of ECL-2 that comprises the amino acid difference. Typically, polypeptide fragments will comprise at least six amino acids.

Also included as polypeptides of the invention are variants and derivatives of the above. The terms "variant" or "derivative" in relation to the amino acid sequences of the present invention includes any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) amino acids from or to the sequence providing that the resulting sequence comprises an ECL-2 region with at least one amino acid difference to the corresponding wild type sequence.

Thus polypeptides of the invention may be modified for use in the present invention. Typically, modifications are made that maintain the biological activity of the sequence. Amino acid substitutions may be made, for example from 1, 2 or 3 to 10, 20 or 30 substitutions. Amino acid substitutions may include the use of non-naturally occurring

analogues, for example to increase blood plasma half-life of a therapeutically administered polypeptide.

Conservative substitutions may be made, for example according to the Table below. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar - uncharged	C S T M
		N Q
	Polar - charged	D E
		K R
AROMATIC		H F W Y

Polypeptides of the invention are typically made by recombinant means, for example as described below. However they may also be made by synthetic means using techniques well known to skilled persons such as solid phase synthesis. Proteins of the invention may also be produced as fusion proteins, for example to aid in extraction and purification. Examples of fusion protein partners include glutathione-S-transferase (GST), 6xHis, GAL4 (DNA binding and/or transcriptional activation domains) and β-galactosidase. It may also be convenient to include a proteolytic cleavage site between the fusion protein partner and the protein sequence of interest to allow removal of fusion protein sequences. Preferably the fusion protein will not hinder the function of the protein of interest sequence. Polypeptides of the invention may also be purified from cells/tissue taken from individuals that naturally express polymorphic forms of PAR-2.

Polypeptides of the invention may be in a substantially isolated form. It will be understood that the protein may be mixed with carriers or diluents which will not interfere with the intended purpose of the protein and still be regarded as substantially isolated. A protein of the invention may also be in a substantially purified form, in which case it will generally comprise the protein in a preparation in which more than 90%, e.g. 95%, 98% or 99% of the protein in the preparation is a protein of the invention.

B. Polynucleotides

Polynucleotides of the invention comprise nucleic acid sequences encoding the polypeptide sequences of the invention. It will be understood by a skilled person that numerous different polynucleotides can encode the same polypeptide as a result of the degeneracy of the genetic code. In addition, it is to be understood that skilled persons may, using routine techniques, make nucleotide substitutions that do not affect the polypeptide sequence encoded by the polynucleotides of the invention to reflect the codon usage of any particular host organism in which the polypeptides of the invention are to be expressed. A particularly preferred polynucleotide of the invention comprises a thymidine residue at position 719 of the sense strand of the coding region of the nucleotide sequence set out in SEQ ID No. 1.

Polynucleotides of the invention may comprise DNA or RNA. They may be single-stranded or double-stranded. They may also be polynucleotides which include within them synthetic or modified nucleotides. A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the polynucleotides described herein may be modified by any method available in the art. Such modifications may be carried out in order to enhance the *in vivo* activity or life span of polynucleotides of the invention.

Where the polynucleotide of the invention is double-stranded, both strands of the duplex, either individually or in combination, are encompassed by the present invention. Where the polynucleotide is single-stranded, it is to be understood that the complementary sequence of that polynucleotide is also included within the scope of the present invention.

Polynucleotides encoding wild type PAR-2 or naturally occurring variants can be obtained in a number of ways. Other variants of the sequences described herein may be obtained for example by probing DNA libraries made from a range of individuals, for example individuals from different populations. In addition, other homologues particularly homologues found in mammalian cells (e.g. hamster, bovine or primate cells), may be obtained by probing cDNA

libraries made from, or genomic DNA libraries from, other animal species, and probing such libraries with probes comprising all or part of known PAR-2 sequences under conditions of medium to high stringency. Similar considerations apply to obtaining species homologues and allelic variants of the known human, rat or mouse PAR-2 sequences.

Variants and strain/species homologues may also be obtained using degenerate PCR which will use primers designed to target sequences within the variants and homologues encoding conserved amino acid sequences within the sequences of the present invention. Conserved sequences can be predicted, for example, by aligning the amino acid sequences from several variants/homologues. Sequence alignments can be performed using computer software known in the art. For example the GCG Wisconsin PileUp program is widely used.

The primers used in degenerate PCR will contain one or more degenerate positions and will be used at stringency conditions lower than those used for cloning sequences with single sequence primers against known sequences.

Alternatively, such polynucleotides may be obtained by site directed mutagenesis of characterised sequences, such as SEQ ID. No 1. This may be useful where for example silent codon changes are required to sequences to optimise codon preferences for a particular host cell in which the polynucleotide sequences are being expressed. Other sequence changes may be desired in order to introduce restriction enzyme recognition sites, or to alter the property or function of the polypeptides encoded by the polynucleotides.

Polynucleotides of the invention may be used to produce a primer, e.g. a PCR primer, a primer for an alternative amplification reaction, a probe e.g. labelled with a revealing label by conventional means using radioactive or non-radioactive labels, or the polynucleotides may be cloned into vectors. Such primers, probes and other fragments will be at least 15, preferably at least 20, for example at least 25, 30 or 40 nucleotides in length, and are also encompassed by the term polynucleotides of the invention as used herein.

Primers and probes may conveniently be used to genotype individuals to establish their PAR-2 status as described below.

Polynucleotides such as a DNA polynucleotides and probes according to the invention may be produced recombinantly, synthetically, or by any means available to those of skill in the art. They may also be cloned by standard techniques.

In general, primers will be produced by synthetic means, involving a step wise manufacture of the desired nucleic acid sequence one nucleotide at a time. Techniques for accomplishing this using automated techniques are readily available in the art.

Longer polynucleotides will generally be produced using recombinant means, for example using a PCR (polymerase chain reaction) cloning techniques. This will involve making a pair of primers (e.g. of about 15 to 30 nucleotides) flanking a region of the sequence which it is desired to clone, bringing the primers into contact with mRNA or cDNA obtained from an animal or human cell, performing a polymerase chain reaction under conditions which bring about amplification of the desired region, isolating the amplified fragment (e.g. by purifying the reaction mixture on an agarose gel) and recovering the amplified DNA. The primers may be designed to contain suitable restriction enzyme recognition sites so that the amplified DNA can be cloned into a suitable cloning vector

C. Nucleotide vectors

Polynucleotides of the invention can be incorporated into a recombinant replicable vector. The vector may be used to replicate the nucleic acid in a compatible host cell. Thus in a further embodiment, the invention provides a method of making polynucleotides of the invention by introducing a polynucleotide of the invention into a replicable vector, introducing the vector into a compatible host cell, and growing the host cell under conditions which bring about replication of the vector. The vector may be recovered from the host cell. Suitable host cells include bacteria such as *E. coli*, yeast, mammalian cell lines and other eukaryotic cell lines, for example insect Sf9 cells.

Preferably, a polynucleotide of the invention in a vector is operably linked to a control sequence that is capable of providing for the expression of the coding sequence by the host cell, i.e. the vector is an expression vector. The term "operably linked" means that the components described are in a relationship permitting them to function in their intended

manner. A regulatory sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under condition compatible with the control sequences.

The control sequences may be modified, for example by the addition of further transcriptional regulatory elements to make the level of transcription directed by the control sequences more responsive to transcriptional modulators.

Vectors of the invention may be transformed or transfected into a suitable host cell as described below to provide for expression of a protein of the invention. This process may comprise culturing a host cell transformed with an expression vector as described above under conditions to provide for expression by the vector of a coding sequence encoding the protein, and optionally recovering the expressed protein.

The vectors may be for example, plasmid or virus vectors provided with an origin of replication, optionally a promoter for the expression of the said polynucleotide and optionally a regulator of the promoter. The vectors may contain one or more selectable marker genes, for example an ampicillin resistance gene in the case of a bacterial plasmid or a neomycin resistance gene for a mammalian vector. Vectors may be used, for example, to transfect or transform a host cell.

Control sequences operably linked to sequences encoding the protein of the invention include promoters/enhancers and other expression regulation signals. These control sequences may be selected to be compatible with the host cell for which the expression vector is designed to be used in. The term promoter is well-known in the art and encompasses nucleic acid regions ranging in size and complexity from minimal promoters to promoters including upstream elements and enhancers.

The promoter is typically selected from promoters which are functional in mammalian, cells, although prokaryotic promoters and promoters functional in other eukaryotic cells may be used. The promoter is typically derived from promoter sequences of viral or eukaryotic genes. For example, it may be a promoter derived from the genome of a cell in which expression is to occur. With respect to eukaryotic promoters, they may be

promoters that function in a ubiquitous manner (such as promoters of a-actin, b-actin, tubulin) or, alternatively, a tissue-specific manner (such as promoters of the genes for pyruvate kinase). They may also be promoters that respond to specific stimuli, for example promoters that bind steroid hormone receptors. Viral promoters may also be used, for example the Moloney murine leukaemia virus long terminal repeat (MMLV LTR) promoter, the rous sarcoma virus (RSV) LTR promoter or the human cytomegalovirus (CMV) IE promoter.

It may also be advantageous for the promoters to be inducible so that the levels of expression of the polynucleotide of the invention can be regulated during the life-time of the cell. Inducible means that the levels of expression obtained using the promoter can be regulated.

In addition, any of these promoters may be modified by the addition of further regulatory sequences, for example enhancer sequences. Chimeric promoters may also be used comprising sequence elements from two or more different promoters described above.

D. Host cells

Vectors and polynucleotides of the invention may be introduced into host cells for the purpose of replicating the vectors/polynucleotides and/or expressing the proteins of the invention encoded by the polynucleotides of the invention. It is preferred to use eukaryotic cells, for example yeast, insect or mammalian cells, in particular mammalian cells to express a polypeptide of the invention.

Vectors/polynucleotides of the invention may introduced into suitable host cells using a variety of techniques known in the art, such as transfection, transformation and electroporation. Where vectors/polynucleotides of the invention are to be administered to animals, several techniques are known in the art, for example infection with recombinant viral vectors such as retroviruses, herpes simplex viruses and adenoviruses, direct injection of nucleic acids and biolistic transformation.

E. Protein Expression and Purification

Host cells comprising polynucleotides of the invention may be used to express proteins of the invention. Host cells may be cultured under suitable conditions which allow expression of the proteins of the invention. Expression of the proteins of the invention may be constitutive such that they are continually produced, or inducible, requiring a stimulus to initiate expression. In the case of inducible expression, protein production can be initiated when required by, for example, addition of an inducer substance to the culture medium, for example dexamethasone or IPTG.

Proteins of the invention can be extracted from host cells by a variety of techniques known in the art, including enzymatic, chemical and/or osmotic lysis and physical disruption.

F. Antibodies

The invention also provides monoclonal or polyclonal antibodies to polypeptides of the invention or fragments thereof. Thus, the present invention further provides a process for the production of monoclonal or polyclonal antibodies to polypeptides of the invention. Preferably, these antibodies are specific for the polypeptides of the invention i.e. have little or no cross-reactivity with wild type PAR-2.

If polyclonal antibodies are desired, a selected mammal (e.g., mouse, rabbit, goat, horse, etc.) is immunised with an immunogenic polypeptide comprising a variant ECL-2 sequence epitope(s). Serum from the immunised animal is collected and treated according to known procedures. If serum containing polyclonal antibodies to a PAR-2 epitope contains antibodies to other antigens, the polyclonal antibodies can be purified by immunoaffinity chromatography. Techniques for producing and processing polyclonal antisera are known in the art. In order that such antibodies may be made, the invention also provides polypeptides of the invention or fragments thereof haptenised to another polypeptide for use as immunogens in animals or humans.

Monoclonal antibodies directed against ECL-2 epitopes in the polypeptides of the invention can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies by hybridomas is well known. Immortal antibody-producing cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. Panels of monoclonal antibodies produced against orbit epitopes can be screened for various properties; i.e., for isotype and epitope affinity.

An alternative technique involves screening phage display libraries where, for example the phage express scFv fragments on the surface of their coat with a large variety of complementarity determining regions (CDRs). This technique is well known in the art.

Antibodies, both monoclonal and polyclonal, which are directed against ECL-2 epitopes are particularly useful in diagnosis. Monoclonal antibodies, in particular, may be used to raise anti-idiotype antibodies. Anti-idiotype antibodies are immunoglobulins which carry an "internal image" of the antigen of the agent against which protection is desired. Techniques for raising anti-idiotype antibodies are known in the art. These anti-idiotype antibodies may also be useful in therapy.

For the purposes of this invention, the term "antibody", unless specified to the contrary, includes fragments of whole antibodies which retain their binding activity for a target antigen. Such fragments include Fv, F(ab') and $F(ab')_2$ fragments, as well as single chain antibodies (scFv). Furthermore, the antibodies and fragments thereof may be humanised antibodies, for example as described in EP-A-239400.

Antibodies may be used in method of detecting polypeptides of the invention present in biological samples by a method which comprises:

(a) providing an antibody of the invention;

(b) incubating a biological sample with said antibody under conditions which allow for the formation of an antibody-antigen complex; and

(c) determining whether antibody-antigen complex comprising said antibody is formed.

Suitable samples include extracts tissues such as brain, breast, ovary, lung, colon, pancreas, testes, liver, muscle and bone tissues or from neoplastic growths derived from such tissues.

Antibodies of the invention may be bound to a solid support and/or packaged into kits in a suitable container along with suitable reagents, controls, instructions and the like.

G. Uses

(i) Genotyping

We have shown that a F240S polymorph of PAR-2 is present at an allelic frequency of 8.4% in a group of 125 individuals, with 15.2% of individuals carrying at least one variant allele. Furthermore, we have shown that the F240S polymorph displays differential responses to PAR-activating agonists compared to wild type PAR-2. The altered properties of the polymorph may be highly significant in terms of drug regimes based on using antagonists that target PAR-2 since antagonists designed to target the wild type PAR-2 protein may be ineffective or indeed deleterious when administered to individuals possessing an S allele. Alternatively, FS heterozygotes and/or SS homozygotes may have increased susceptibility to diseases, such as inflammatory diseases. Consequently, determination of an individual's PAR-2 status may be used to identify individuals at risk from diseases associated with variant PAR-2.

Thus, it will be highly desirable to establish the genotype of any individual to whom PAR-2 antagonists are to be administered to determine whether they possess an S allele or any other similar variation.

Accordingly, the present invention provides a method of determining the PAR-2 genotype of an individual which method comprises determining all or part of the sequence of the ECL-2 region within the individual's PAR-2 genes. Preferably, the sequence of the nucleotide codon that specifies amino acid 240, or its equivalent, is determined, more preferably the sequence of the polymorphic site at nucleotide 719 is determined.

The sequence may be determined for genomic DNA or mRNA, preferably genomic DNA. A variety of techniques for doing this are known in the art such as RFLP analysis or direct genomic sequencing – both of which are described in the Examples. Determination of the sequence in the present context does not necessarily mean that the exact nucleotide sequence be established since, for example, the presence or absence or a restriction fragment of a particular size can be sufficient (see the Examples).

Nucleic acids from the individual of interest are typically obtained from a biological sample taken from the individual, such as a blood sample or other tissue sample.

Typically, genotyping may be performed using PCR primers that flank the ECL-2 region. Accordingly, the present invention provides a pair of oligonucleotide primers, each comprising at least 10 contiguous nucleotides of the PAR-2 nucleotide sequence and which flank the ECL-2 region. These primer pairs are provided for use in establishing the PAR-2 genotype of an individual. PCR primers may be packaged into kits together with other components such as buffers, instructions etc.

Genotyping may also be carried out using a protein-based approach, for example using antibodies to PAR-2 polypeptides of the invention (see section F).

(ii) Assays for identifying agonists/antagonists of variant PAR-2 polypeptides

As discussed above, the F240S PAR-2 polymorph has different properties to the wild type PAR-2 such that agonists/antagonists that may be used to affect the activity of the wild type PAR-2 receptor may not be effective against the polymorph. Accordingly, it would be desirable to identify agonists/antagonists that are effective in those patients possessing at least one S allele or other polymorph having altered properties compared with wild type.

Accordingly, the present invention provides screening methods for identifying compounds that modulate the activity of a polypeptide of the invention, for example stimulate or inhibit a polypeptide of the invention.

In one type of easily conducted *in vitro* assay, candidate compounds can be tested for binding to a polypeptide of the invention. Binding assays are well known in the art. In a preferred embodiment, candidate compounds are tested in competition assays with a known agonist or antagonist. The agonist or antagonist may be labeled with a detectable label. In one typical protocol, varying concentrations of the candidate compound are supplied along with a constant concentration of labeled agonist or antagonist and the inhibition of a binding of labeled agonist/antagonist to the receptor can be evaluated using known techniques.

More sophisticated functional assays comprise contacting a candidate substance with a polypeptide of the invention and determining whether the polypeptide is stimulated or inhibited. In the case of testing for agonists that stimulate a polypeptide of the invention, one suitable assay is the calcium signaling assay described in the Examples and reference 31. Thus, for example the effect of candidate compounds on agonist-induced responses can be measured in the cells recombinantly expressing a variant PAR-2 receptor of the inventions.

Assay systems for the effect of activation of receptor on these cells include calcium mobilization and voltage clamp which are described herein in further detail. These assays permit an assessment of the effect of the candidate compound on the receptor activity rather than simply the ability to bind to the receptor. Agonist-induced increases Ca release by cells expressing variant PAR-2 receptors can be assessed using known techniques. One such protocol is described in US Patent No. 5,874,400, incorporated herein by reference. Using these types of assays, the ability of a candidate compound to activate the receptor can be tested directly. In an alternative to measuring calcium mobilization, the voltage clamp assay can be used as a measure for receptor activation. Agonist-induced inward chloride currents are measured in voltage-clamped cells expressing variant PAR-2 receptors essentially as previously described (Julius et al, Science (1988) 241:558-563) except that the single electrode voltage-clamp technique is employed.

Other assays involve measuring cytokine release from keratinocytes (16) and the rolling and adhesion of leukocytes to the endothelium.

Antagonists can be tested in a similar manner except that a known agonist is used alone in the control samples and coadministered to cells along with the candidate compound in the test samples.

Suitable known agonists include trypsin, tryptase, TFLLR-NH2, trans-cinnamoyl-LIGRLO-NH_2 together with a number of agonists described in US Patent No. 5,874,400. Suitable known antagonists for use in the assay methods of the invention are also described in US Patent No. 5,874,400.

In one aspect, it may be preferred to identify modulators of the activity of polypeptides of the invention that are specific for those peptides and have little or no affect on wild type PAR-2.

In another aspect, it may be preferred to identify modulators of the activity of polypeptides of the invention that also modulate wild type PAR-2.

Thus assays of the invention may further include the step of determining the effect of an agonist or antagonist of a polypeptide of the invention on wild type PAR-2, typically using similar assay techniques.

Candidate compounds of the invention may also be tested using an animal model to determine whether, for example, they can affect inflammatory responses caused by the administration of PAR-2 agonists (see reference 21).

One such animal model is described in US Patent No 5,874,400 where an agonist is injected into the femoral vein of a rat and the arterial pressure monitored. This assay may be used to assay candidate agonists by administered a candidate compound in place of the known agonist, or to assay candidate antagonists by coadministering candidate compound with the known agonist. The effect of candidate compounds can also be measured by observing vasodilation as a result of stimulation of the femoral vein as described in US Patent No 5,874,400.

Candidate compounds

Suitable candidate substances include peptides, especially of from about 5 to 30 or 10 to 25 amino acids in size, based on the sequence of the PAR-2, in particular the tethered ligand sequences present in PAR-2 or variants of such peptides in which one or more residues have been substituted. Other peptide candidates include the peptide agonists and antagonists described in US Patent No. 5,874,400 or variants of such peptides in which one or more residues have been substituted. Peptides from panels of peptides comprising random sequences or sequences which have been varied consistently to provide a maximally diverse panel of peptides may be used.

Suitable candidate substances also include antibody products (for example, monoclonal and polyclonal antibodies, single chain antibodies, chimeric antibodies and CDR-grafted antibodies) which are specific for a polypeptide of the invention. Furthermore, combinatorial libraries, peptide and peptide mimetics, defined chemical entities, oligonucleotides, and natural product libraries may be screened for activity as modulators of PAR-2 activity. The candidate substances may be used in an initial screen in batches of, for example 10 substances per reaction, and the substances of those batches which show inhibition tested individually. Candidate substances which show activity in *in vitro* screens such as the competition binding assays described assays can then be tested in whole cell systems, also described above.

H. Therapeutic uses

Antagonists/agonists of polypeptides of the present invention may be used to treat more effectively patients suffering from various disorders, which patients possess one or more PAR-2 polymorphs according to the present invention. For example, antagonists of polypeptides of the present invention may be used to treat inflammatory diseases or conditions characterized by inflammation for example, asthma, chronic obstructive pulmonary disease, arthritis, inflammatory bowel disease, psoriasis and eczema. They may also be used to treat disorders such as multiple sclerosis and to raise blood pressure.

By contrast, agonists of the polypeptides of the invention may, for example, be used as antihypertensives.

Compounds of the invention that modulate the activity of a polypeptide of the invention, for example substances identified or identifiable by the assay methods of the invention, may preferably be combined with various components to produce compositions of the invention. Preferably the compositions are combined with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition (which may be for human or animal use). Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline.

The composition of the invention may be administered by direct injection. The composition may be formulated for parenteral, intramuscular, intravenous, subcutaneous, oral or transdermal administration. More recently, alternative means for systemic administration of peptides have been devised which include transmucosal and transdermal administration using penetrants such as bile salts or fusidic acids or other detergents. In addition, if properly formulated in enteric or encapsulated formulations, oral administration may also be possible. Administration of these compounds may also be topical and/or localized, in the form of salves, pastes, gels and the like.

Typically, each compound may be administered at a dose of from 0.01 to 30 mg/kg body weight, preferably from 0.1 to 10 mg/kg, more preferably from 0.1 to 1 mg/kg body weight.

The routes of administration and dosages described are intended only as a guide since a skilled practitioner will be able to determine readily the optimum route of administration and dosage for any particular patient and condition.

The present invention will now be illustrated with references to the following examples that are non-limiting

Description of the figures

Figure 1. Comparison of the nucleotide sequences of the two human PAR-2 alleles. Sequence analysis was performed on a cohort of Caucasian subjects in order to confirm the existence of the two forms of PAR-2. Subjects (a) homozygous for 719-T, or (b) homozygous for 719-C are shown and (c) heterozygous for 719-T/C (where Y = T or C). The codon of the amino acid 240 modified by the mutation is represented is underlined.

Figure 2. Location of the polymorphism and alignment of the ECL-2 amino acid residues from mouse, rat and human PAR-2. Note the conservation at the position of the Phe240 and neighboring amino acid residues. Amino acid numbers are from the human receptor.

Figure 3. Calcium signaling in the human wild-type and PAR_2F240S receptors in response to trypsin and PAR2-APs. (a) trypsin; (b) SLIGKV-NH_2; (c) tc-NH_2; and (d) SLIGRL-NH_2. Wild type responses are designated by: —◆—, and PAR_2F240S, - -Δ- -. Cells were lifted with non-enzymatic cell dissociation fluid, loaded with Fluo-3 (22μM) prior to incubation for 35min at RT. Cells were challenged with different concentrations PAR agonists and responses were monitored by fluorescence spectrophotometry (excitation 480nm, emission 530nm). Responses were normalized to the peak height obtained with 2 μM calcium ionophore (% A23187). Each data point represents the mean ± SEM of three to four separate experiments each performed in duplicate.

Figure 4. Calcium signaling for the human wild-type and PAR_2F240S receptors in response to the PAR-1APs SFLLR-NH_2, CitNH_2 and TFLLR-NH_2.

(a) Concentration effect curves to SFLLR-NH_2 and Cit-NH_2; Wild type response to SFLLR-NH_2, —◆—; PAR_2F240S, - -Δ- -. Wild type response to Cit-NH_2, —★—; and PAR_2F240S, ---⊕---

(b) Concentration effect curves to TFLLR-NH_2. Wild type response to TFLLR-NH_2, —◆—; and PAR_2F240S, - -Δ- -. Cells were lifted with non-enzymatic cell dissociation fluid, loaded with Fluo-3 (22 μM) prior to incubation for 35min at RT. Cells were challenged with different concentrations PAR-1APs and responses were monitored by fluorescence

spectrophotometry (excitation 480nm, emission 530nm). Responses were normalized to the peak height obtained with 2 μM calcium ionophore (% A23187). Each data point represents the mean ± SEM of three to four separate experiments performed in duplicate, grown from individually grown crops of cells.

Figure 5. *Calcium signaling in human and rat wild-type and PAR_2F240S Receptors in response to TLIGRL-NH2 and tc-YGPKF-NH_2.* Cells were lifted with non-enzymatic cell dissociation fluid, loaded with Fluo-3 (22 μM) prior to incubation for 35min at RT. Cells were challenged with the agonists shown and responses were monitored by fluorescence spectrophotometry (excitation 480nm, emission 530nm). Responses were normalized to the peak height obtained with SFLLR-NH_2 (50 μM).

EXAMPLES

Materials and Methods

Peptides and other Reagents

All peptides were synthesized by solid-phase methods at the Peptide Synthesis Facility, University of Calgary, Faculty of Medicine, Calgary, Alberta, Canada. The nature and purity of the peptides was assessed by HPLC analysis, mass spectral analysis, and quantitative amino acid analysis. Peptides were prepared in 25mM HEPES buffer, pH 7.4 and were standardized by quantitative amino acid analysis to confirm peptide concentration and purity. The pGEM-T-Easy vector, SAU 96 I, taq polymerase, deoxy neucleic triphosphates (dNTPs), MgCl, and 10x PCR reaction buffer were purchased from Promega, Southampton, UK. All Oligonucleotides were synthesized at Oswel Laboratories, Southampton, UK; FCS, DMEM, non enzymatic cell dissociation fluid, penicillin, streptomycin, amphotericin, sodium pyruvate, and PBS (without calcium and magnesium) were from Gibco BRL, Gaithersburg, MD, U.S.A.; Porcine pancreatic type IX trypsin, sulphinpyrazone, and calcium ionophore (A23187) were from Sigma, St Louis, MO. and pcDNA3.1(+) and Geneticin were from Invitrogen, Netherlands.

Cloning of PAR-2 and the PAR-2 Polymorphism

Human colon tissue resected at surgery from patients with colonic cancer was used as a source of RNA. Briefly, freshly collected colon tissue was homogenized before addition of Trizol reagent (Gibco, Paisley, Scotland, UK) and chloroform. The RNA was precipitated at –20°C in isopropanol overnight. The RNA pellet was recovered by centrifugation at 4°C, washed in 80 % ethanol, air dried and suspended in DEPC-treated water and quantified spectrophotometrically at 260 nm. One microgram of total cellular RNA was reverse transcribed (RT) by AMV reverse transcriptase at 42°C for 1 hr using poly $d(T)_{15}$ as a primer. Specific oligonucleotides to sequences at the beginning of (1U-sense 5'-CCA GGA GGA TGC GGA GC-3') and the end of the PAR-2 reading frame (1D-antisense 5'-GAG GAC CTG GAA AAC TCA ATA-3') were used to amplify the cDNA. A PCR product of the expected size (~1200bp) was cloned into the pGEM-T Easy vector using a ligation kit (Promega, Southampton, UK). The product was then sequenced on both strands using the dideoxynucleotide sequencing method (32), employing a ThermoSequence DNA polymerase sequencing kit (Amersham Life Sciences, Bucks, UK). The cDNA encoding human PAR-2 was subcloned into pcDNA3.1(+), amplified using p Bluescript (Stratagene, Cambridge, UK) and purified using a Qiagen maxiprep kit (Qiagen Ltd, Hertfordshire, UK). The product was sequenced to ensure correct orientation of the reading frame.

To obtain a copy of the wild type cDNA sequence of PAR-2 (5), site directed mutagenesis using the QuikChange kit (Stratagene, Cambridge, UK) was performed according to the manufacturers' instructions. The clone was then sequenced for confirmation of the nucleotide change from C719 to T719. To establish whether the Phe240 residue was of importance for the general mechanism of PAR-2 activation, or was just a specific mutation that interfered with human PAR-2 functioning, we performed site directed mutagenesis on a Rat PAR-2 clone (8) mutating the T719 to C719. The clone was then sequenced to confirm the presence of the mutation.

Restriction Fragment Length Polymorphism Analysis (RFLP).

Genomic DNA was extracted from the blood of 125 normal Caucasian individuals by standard techniques (33). Specific PAR-2 primers corresponding to nucleotides 699-718 in extracellular loop II (T2D-sense 5'-GCT CTT GGT GGG AGA CAG GT-3') and

nucleotides 926-949 in extracellular loop III (T4U-antisense 5'-GGC TCT TAA TCA GAA AAT AAT GCA-3') were used to generate a PCR product of 250 bp from the genomic DNA samples. A SAU 96 I site was designed into the T2D-sense primer and was accomplished by replacing nucleotide 716 (T) for G (see primer T2D above, G(716) is underlined). A control Sau96I restriction site was present 100 bp from the 3' end of the PCR product. The PCR reaction was performed in a Gene Amp 2400 PCR System with 300 ng DNA, 150 ng of each primer, 200 μM dNTP's, 2.5 mM MgCl, and 1 U *Taq* polymerase in a 50 μl reaction volume starting at 94°C for 5 min, 35 cycles of denaturing at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 5 min. The resulting PCR product of 250 bp was digested overnight at 37°C using 6U/reaction of Sau 96 I. The products were run on an acrylamide gel, stained in ethidium bromide for 5 min and observed by illumination under ultra violet light. The presence of a 150 bp and 100 bp band signified the absence of the polymorphic alleles, whilst the presence of a 130 bp and 100 bp band in the absence of the 150 bp band demonstrated the presence of both polymorphic alleles.

Genomic DNA Sequencing.

To confirm data obtained from the RFLP analysis, oligonucleotides corresponding to nucleotides 389-409 in extracellular loop I (S1D-sense 5'-TGA AGA TTG CCT ATC ACA TAC-3') and nucleotides 926-949 in extracellular loop III (T4U-antisense 5'-GGC TCT TAA TCA GAA AAT AAT GCA-3') were used to amplify human genomic DNA. The PCR reaction was performed as described in the RFPL analysis except the annealing temperature was set to 54°C. The 560 bp products containing the polymorphic nucleotide site, 719, were purified using a PCR purification kit (Qiagen) before fluorescence-based automated cycle sequencing was performed on an ABI 377 (ABI PRISM™ Dye Primer Cycle Sequencing-21M13 FS and M13REV FS Ready Reaction Kits).

Cell Culture and Transfection. Kirsten sarcoma transformed rat kidney epithelial cells (KNRK, American Tissue Type Culture Collection, Bethesda, MD, U.S.A) in fresh medium (DMEM, 5 % FCS (vol/vol), 100 μM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B) were seeded into 60 mm petri dishes and incubated overnight in 95 % air, 5 % CO_2 at 37°C. At 60 % confluence, transfection was performed using the Lipofectamine method according to the manufactuerer's protocol

(Gibco/BRL) with 8 μg of each construct per petri dish. Transfected cells were subcloned in geneticin (0.6 mg/ml) containing medium and clones were initially selected by their ability to produce a calcium signal in response to trypsin and the PAR-2 selective peptide tc-NH_2. To obtain permanent cell lines for each receptor, cells expressing high levels of PAR-2 were isolated by fluorescence-activated cell sorting using the B5 anti-PAR-2 polyclonal rabbit antibody (as described elsewhere (34)). All cell lines were routinely propagated in geneticin containing medium without the use of trypsin in 95 % air, 5 % CO_2 at 37°C. The rat PAR-2 cell line has been described elsewhere (8,31). Clones with matched expression, as assessed by FACS analysis were selected for functional studies.

Calcium Signaling Assay.

The calcium cell-signaling assay was performed as described previously (31). Briefly, cells were seeded in 80 cm^2 flasks (Gibco/BRL) and incubated until 90 % confluence was achieved. Following two washes with PBS (without calcium and magnesium) cells were incubated with 5 ml of non-enzymatic cell dissociation fluid, and centrifuged at 200 g for 10 min. The cell pellet was resuspended in 1 ml of DMEM, 10 % FCS and 0.25 mM sulphinpyrazone. To the cell suspension, 10 μl of 2.5 mg/ml of Fluo-3 acetoxymethyl ester (Molecular Probes Inc., Eugene, OR) was added prior to gentle shaking for 35 min at room temperature. Cells were then washed in calcium assay buffer (150 mM NaCl, 3 mM KCl, 1.5 mM $CaCl_2$, 10 mM glucose, 20 mM HEPES, 0.25 mM sulphinpyrazone, pH 7.4) to remove excess dye and resuspended in 1.5 ml of calcium assay buffer. Fluorescence measurements were performed on a Perkin-Elmer fluorescence spectrometer (LM 150?), with an excitation wavelength of 480 nm and emission recorded at 530 nm. Cell suspensions (2 ml of 3 x 10^5 cells/ml) in 4 ml cuvettes were stirred with a magnetic flea bar and maintained at 24°C. To establish concentration effect curves, the signal produced by the addition of a test agonist was measured as a percentage of the fluorescence peak height yielded by the addition of 2 μM calcium ionophore. Previous experiments had established that a plateau of the dose response curve was obtained with 2 μM calcium ionophore. Addition of the protease inhibitor (Amastatin) had no effect on the response of the test peptides, therefore protease inhibitors were omitted from the experimental procedure.

Results

Cloning of the Human PAR-2 receptor.

The reading frame for PAR-2 was cloned from cDNA derived from human colon tissue using specific primers synthesized on the basis of the 5' and 3' extremities of the published sequence (4). A comparison of the obtained sequence with the published sequence revealed a transition from a Thymine to a Cytosine at nucleotide position 719. Initially we set out to establish whether the mutation was a PCR artifact or existed within a normal population. Therefore we sequenced a selection of normal individuals from a Caucasian population and confirmed the existence of the mutation by DNA sequencing (Figure 1). Individuals homozygous for wild type PAR-2 were identified by a single thymine residue at position 719 (Figure 1a), whilst individuals homozygous for polymorphic PAR-2 where identified by the presence of a cytosine at residue 719 (Figure 1b). Finally, heterozygous individuals were identified by the presence of both a thymine and a cytosine at residue 719 (Figure 1c). This mutation is predicted to induce an amino acid change from phenylalanine 240 (F240) to serine 240 (S240). The site of the mutation is approximately six amino acids from the fifth transmembrane domain, in ECL-2 (Figure 2)

Genotyping of the PAR-2 polymorphism.

RFPL analysis and automated DNA sequencing were employed to determine the frequency of the polymorphism in a Caucasian population. The frequency of the homozygous S/S genotype was found to be relatively uncommon representing less than 2% from the cohort of individuals examined (Table 1). The data were found to agree with the Hardy-Weinberg equilibrium ($F^2 + 2FS + S^2 = 1$; F^2 = homozygotes F/F frequency, S^2 = homozygotes S/S frequency and 2FS = heterozygotes F/S frequency), indicating that the two alleles of the PAR-2 gene are segregated in a Mendelian manner.

Table 1. *Distribution of the PAR-2 alleles in a Normal Caucasian Population.* Restriction fragment length polymorphism analysis and direct sequencing of genomic DNA (see methods) were used to genotype 125 normal individuals for PAR-2 alleles [nucleotide 719T (240F) and/or nucleotide 719C (240S)]. Allele and gene frequencies are shown.

	Subjects
	n = 125
Genotype	
No. of subjects (% of group)	
240 F/F	106 (84.8%)
240 F/S	17 (13.6%)
240 S/S	2 (1.6%)
Allelic frequency	
240 F	0.916
240 S	0.084

PAR_2F240S is less sensitive to the PAR-2 agonists Trypsin, SLIGKV-NH_2 and SLIGRL-NH_2, but more sensitive to tc-NH_2.

To ascertain whether the F240 to S240 mutation altered the response of the receptor to its agonists, we expressed both the wild-type and PAR_2F240S as permanent KNRK cell lines with matching receptor expression as assessed by cell surface fluorescence (FACS) analysis. These cell lines were then used to study receptor agonist activation responses as monitored by elevations of intracellular calcium.

The concentration effect curves for trypsin and several PAR-2 selective activating peptides are shown in Figure 3. For the wild-type receptor, trypsin was 3 orders of magnitude more potent than the PAR-2AP, SLIGKV-NH_2, tc-NH_2 and SLIGRL-NH_2, and the relative rank order of potencies were trypsin>>>>tc-NH_2≥SLIGRL-NH_2>SLIGKV-NH_2. This order of agonist potencies is in keeping with a previous report (31). Trypsin stimulated a detectable calcium elevation at 1 nM reaching a near maximum response at 100 nM. The effective response to the PAR-2AP tc-NH_2, was between 1 - 100 μM, SLIGKV-NH_2 from 5 - 200 μM and SLIGRL-NH_2 was effective from 2-100 μM (Figure 3a, b, c, and d respectively). For the PAR_2F240S receptor, the potencies of all the PAR-2 agonists were significantly different (Figure 3). Trypsin was nearly 4 fold less potent (Figure 3 and Table 2), activating the PAR_2F240S receptor between 5 and 500 nM, resulting in a rightward shift in the concentration-effect curve (Figure 3a). The parent tethered ligand peptide, SLIGKV-NH_2, was also found to be less potent in this system by over 2 fold, activating the PAR_2F240S receptor between 10 and 500 μM (Figure 3b and Table 2). However, in both systems the maximal response to SLIGKV-NH_2 appeared be lower than the maximal response obtained with the other PAR-2 agonists (~50 % versus ~60% relative to calcium

ionophore). The tc-NH_2 peptide was 4 fold more potent (Figure 3c and Table 2), activating the PAR_2F240S receptor between 0.2 and 20 μM compared to 1 and 50 μM in the wild-type receptor. The potency of SLIGRL- NH_2 was reduced by a similar degree to that of SLIGKV-NH_2 (~ 2.5 fold) activating PAR_2F240S between 5 and 200 μM (Figure 3 and Table 2). The relative order of potencies for these agonists in the PAR_2F240S system which differ quantitatively from those for the wild-type receptor were: trypsin>>tc-NH_2>>SLIGRL-NH_2>SLIGKV-NH_2.

PAR_2F240S is More Sensitive to the Selective PAR-1 Agonist TFLLR-NH_2 but Displays no Difference in Sensitivity to SFLLR-NH_2 and Cit-NH_2.

Given that the PAR-1AP SFLLR-NH_2 can activate PAR-2 (9,10), we sought to investigate whether the PAR_2F240S receptor exhibited any difference in reactivity towards this human PAR-1 derived peptide, in addition to possible differences to two other selective PAR-1APs, TFLLR-NH_2 and Cit-NH_2. For the wild-type receptor, the relative rank order of potencies were SFLLR-NH_2>Cit-NH_2>TFLLR-NH_2 as previously reported (35). SFLLR-NH_2, Cit-NH_2 and TFLLR-NH_2 were found to stimulate receptor activation between 5 - 200 μM, 50 - 400 μM and 100 – 800 μM respectively (Figure 4a and Table 2). In the PAR_2F240S receptor SFLLR-NH_2 and Cit-NH_2 were found to induce a response between 5 – 100 μM and 50 - 400μM as was found in the wild-type receptor. Surprisingly, TFLLR-NH_2, developed as a PAR-1 selective agonist, was found to be over 7 fold more potent in the PAR_2F240S receptor, provoking a response from 10 – 400 μM (Figure 4b and Table 2). The relative rank order of potencies for these PAR-1AP in the PAR_2F240S receptor SFLLR-NH_2>TFLLR-NH_2>Cit-NH_2 differed qualitatively from those for the wild-type receptor above). Following desensitization of PAR_2F240S by the prior addition of the PAR-2 selective peptide tc-NH_2 (50 μM), we observed that TFLLR-NH_2 (50 μM) was without effect (data not shown). Furthermore, thrombin at 5 U/ml (a concentration shown to be maximal for PAR-1 activation (35)) failed to induce a calcium response in both the wild-type and PAR_2F240S cell lines (data not shown).

	Relative receptor Sensitivity	
Agonist	*Human PAR_2F240S*	*Rat PAR_2F240S*
PAR-2 Agonists		
SLIGKV-NH_2	2.50	ND
tc-LIGRLO-NH_2	0.25	0.29
SLIGRL-NH_2	2.79	2.8
Trypsin (x 10^3)	3.65	1.7
PAR-1 Agonists		
Cit-A	1.24	ND
SFLLR-NH_2	0.084	ND
TFLLR-NH_2	0.14	0.29

Table 2. *Ratio (Polymorphic/Wild) of Agonist Potencies Between the PAR-2S and PAR-2F Receptor Systems.* The activities of each agonist in the PAR-2S receptor system was expressed relative to the activity of each agonist in the PAR-2F receptor, according to the equation: $R_{EC,\ PAR2}=EC_{PAR2F240S}/EC_{wild\text{-}type}$. The concentration of each agonist causing a response in the PAR_2F240S receptor ($EC_{PAR2F240S}$) was divided by the concentration of that agonist required to cause the same calcium signal (relative to A23187) in the wild-type receptor ($EC_{wild\text{-}type}$). Values are averages obtained from four points along the parallel region of the dose response curves shown in Figures 3 and 4. Values greater than 1.0 designate a sensitivity that is lower in the PAR_2F240S receptor than in the wild-type receptor.

Rat-PAR_2F240S Displays Identical Changes in Agonist Sensitivity as the Human PAR_2F240S.

To determine whether the F240 residue is important for PAR-2 agonist specificity, we performed site-directed mutagenesis in rat-PAR-2, mutating F240 to S240. A permanent cell line in KNRK cells was established. The relative potencies for the PAR-activating agonists on the rat-PAR_2F240S receptor are displayed in Table II. In the rat-PAR_2F240S receptor, trypsin was ~2 fold less potent in the rat-PAR-2 receptor system provoking a response between 1 – 20 nM,(data not shown). In addition SLIGRL-NH_2 was found to be ~3 fold less potent in this system. Tc-NH_2 was 3 fold more potent in the rat-PAR_2F240S receptor as was TFLLR-NH_2.

TLIGRL-NH_2 and tc-YPGKF-NH2 can selectively activate PAR_2F240S but not the wild-type PAR-2 receptor.

We were interested in determining whether substituting a threonine for the serine in SLIGRL-NH_2 would increase the potency of the peptide. Furthermore we tested the PAR-4 peptides GYPGKF-NH_2 and an activating peptide, tc-YPGKF-NH_2 designed on the basis of the activating sequence of PAR-4. The results are shown in Figure 5. In both human and rat PAR_2F240S cell lines TLIGRL-NH_2 (50 μM) displayed a significant increase in activity. In the human wild-type receptor, TLIGRL-NH_2 (50 μM) was virtually without effect, however TLIGRL-NH_2 (50 μM) provoked a response similar to that of SFLLR-NH_2 (50 μM) in the PAR_2F240S receptor. In the rat wild type receptor TLIGRL-NH_2 (50 μM) stimulated a small response, but this response was half that observed in the rat-PAR_2F240S cell line. The PAR-4 peptide GYPGKF-NH_2 (400μM) was without effect in rat and human wild-type and PAR_2F240S systems (data not shown). The tc-YGPKF-NH_2 peptide was found to have no effect in either of the rat or human wild-type cell lines (Figure 5d), but stimulated a robust response equivalent to SFLLR-NH_2 in the human PAR_2F240 system and 50% of the SFLLR-NH_2 response in the rat PAR_2F240S system. Following desensitization of PAR_2F240S by the addition of tc-NH_2 (50μM), TLIGRL-NH_2 (50μM) or tc-YGPGKF-NH_2 (100μM) were without effect (data not shown). Furthermore, addition of TLIGRL-NH_2 (100μM) ablated the response by a submaximal dose (20μM) of SLIGRL-NH_2 (data not shown).

Discussion

The main finding of this study is the discovery of a human PAR-2 genetic polymorphism that displays differential activation in response to trypsin and other PAR agonists. The PAR_2F240S receptor displayed reduced sensitivity to trypsin, SKLIGV-NH_2 and SLIGRL-NH_2, whilst an increase in sensitivity to tc-NH_2 and surprisingly the PAR-1 selective agonist TFLLR-NH_2. In addition we found that the peptide TLIGRL-NH_2 can be used selective agonist of PAR_2F240S. Furthermore, constructing the same mutation in rat-PAR-2 echoed these findings suggesting that F240 may participate directly in regulating agonist specificity. We conclude that the changes in trypsin and other agonist potencies

will have important implications for the development of PAR-2 antagonists and raise the possibility that this polymorphism may be implicated in disease.

Direct DNA sequencing and RFLP analysis confirmed the presence of the polymorphism in a normal human population. Amongst the 125 patients screened, only two were found to be homozygous for the S240 allele, indicating the possibility that individuals expressing this genotype maybe associated with a disease state. Analysis of the data using the Hardy-Weinberg equation indicated that the alleles were normally distributed and are segregated in a Mendelian fashion. Unfortunately, due to patient confidentiality we were unable to contact patients of potential interest to explore their possible phenotypes or association with disease. Nevertheless, separate studies are currently underway to examine the possible links of this polymorphism with a number of disease states.

To study if this polymorphism had a direct impact on PAR-2 signaling, we generated two permanent transfected cell lines either expressing the wild-type or PAR_2F240S receptors. Cell lines expressing comparable receptor densities (as assessed by FACS analysis) were carefully chosen to ensure that differences in ligand activity were attributable to alterations in receptor function and not to differences in receptor densities. Using elevations of calcium as an indicator of receptor activation, we observed that trypsin was approximately 4 fold less potent at provoking a response from the PAR_2F240S receptor. In agreement with the apparent loss of receptor sensitivity towards the tethered ligand was the finding that SKLIGV-NH_2 and SLIGRL-NH_2 were also less potent (about 2.5 and 2.8 fold respectively). The loss of potency by trypsin might occur due to the exposed tethered ligand docking less efficiently onto the body of the receptor as a result of a conformational change induced by the phenylalanine to serine mutation. Alternatively, the tethered ligand may dock efficiently onto the receptor but fail to induce a comparable conformational change, and hence activation, as that induced in the wild-type receptor. In other studies, mutating $P^{231}E^{232}E^{233}$ in ECL-2 of rat PAR-2 to $P^{231}R^{232}R^{233}$ resulted in a dramatic loss of receptor activity towards PAR-2AP, although the potency of trypsin was far less affected by the multiple amino acid substitution (31). Finding that trypsin lost greatest potency compared to the SKV-NH_2 and SRL-NH_2, suggests that Phe240 may play an important role in agonist specificity for both the tethered ligand and the synthetic peptides.

In stark contrast to our observations with trypsin, SLIGKV-NH_2, SLIGRL-NH_2 and other reports where mutations have been constructed in PAR (27,31), was the finding that tc-NH_2 was 4 fold more potent at activating the PAR_2F240S receptor. The main structural difference between the tc-NH_2 compound and the other PAR-2AP (including the natural tethered ligand) is the attached large aromatic *trans*-cinnamoyl group. The loss of the benzene group from the F240 to S240 mutation may allow the *trans*-cinnamoyl group to dock more efficiently thereby inducing greater receptor activation. As binding assays for PAR prove problematic, it is difficult to assess whether the differential agonist potencies observed are due to a change in ligand affinity or efficacy. However, it would be reasonable to argue that the intrinsic activity of the agonists tested are unaffected, as maximal responses for all peptides were similar in both cell lines. Nevertheless, substitution of the large aromatic F240 for a small non-aromatic S240 appears to significantly alter the receptor's sensitivity towards PAR-2 agonists.

We extended our studies to look at the possible shifts in potencies of PAR-1AP. It is well established that the PAR-1 tethered ligand sequence, SFLLR can activate PAR-2, whilst the PAR-2 peptide sequence SLIGKV-NH_2 is without effect on PAR-1 (9,10). Of most interest was the finding that TF-NH_2 showed an increase in potency of over 7 fold resulting in a shift in the rank order of relative potencies between TFLLR-NH_2 and Cit-NH_2. However, we noted that the SFLLR-NH_2 and Cit-NH_2 possessed equal potency in both the wild-type and PAR_2F240S cell lines. These results would suggest that SFLLR-NH_2 and Cit-NH_2 probably interact with ECL-2 of PAR-2 in a different manner to other PAR-2AP. Indeed it has been reported before that mutating ECL-2 of rat-PAR-2 wherein a 15-residue sequence is identical to PAR-1, SFLLR-NH_2 exhibits reduced potency (31). It was also concluded from these studies that SFLLR-NH_2 might interact in a manner distinct from other PAR-2AP. The concentration effect curves obtained with SFLLR-NH_2 and Cit-NH_2 also support the FACS results that receptor densities were of comparable levels on both cell lines, as shifts in potency were not observed.

The reason for the apparent shift in TFLLR-NH_2 and not SFLLR-NH_2 is intriguing. In the light of the data obtained with the TFLLR-NH_2 peptide and noting the similarity in sequences between SFLLR-NH_2 and TFLLR-NH_2, one would have predicted SFLLR-NH_2 to be more potent at activating the PAR_2F240S receptor. It has been previously reported

that substitutions of the serine residue at position one of SLIGKV-NH_2 and SFLLR-NH_2 are not well tolerated for successful activation of PAR-2 (9,10). Indeed, we have found that by substituting the serine in SLIGRL-NH_2 to a threonine generates a peptide (TLIGRL-NH_2) that is selective for our PAR_2F240S receptor, as TLIGRL-NH_2 has reduced potency on the wild type PAR-2 and has no effect on PAR-1 (10). It would appear that PAR_2F240S can tolerate conservative changes at position one of the activating petide, thereby providing convincing evidence this receptor may interact with peptide agonists in a different manner to that of the wild-type receptor. With this in mind, and given that TFLLR-NH_2 has become a useful tool for selectively activating PAR-1 in the presence of PAR-2 (35), we would suggest that data derived with this peptide in bioassays using human tissue or cells should be analyzed with some caution.

After studying the results obtained with the human PAR_2F240S receptor we contemplated whether F240 was an important residue in defining agonist specificity of the PAR-2 receptor in other species, or whether its influence on specificity was restricted to human PAR-2. We therefore constructed the same F240 to S240 mutation in rat-PAR-2. The protein sequence of rat and mouse-PAR-2 shows a high degree of homology with human PAR-2 especially within ECL-2 (36,37). In rat-PAR-2, the F240 residue is conserved, although a serine is present at residue 241 in place of the Asparagine in human PAR-2. We found that in rat-PAR_2F240S the relative shift in agonist potencies echoed those observed with human PAR-2S. Trypsin and SLIGRL-NH_2 were less potent, and as found in the human system tc-NH_2 was more potent. Furthermore, we also noted that TFLLR-NH_2 displayed increased potency in the rat- PAR_2F240S cell line, confirming our earlier observations with the human PAR_2F240S receptor. These results strongly imply that the F240 residue not only serves as an important residue in agonist specificity but also suggests it maybe involved in the underlying mechanism that this receptor employs to identify specific agonists.

Although the PAR-4 peptide GYPGKF-NH_2 was without effect on the rat and human cell lines, we were surprised to find that tc-YGPKF-NH_2 could activate both human and rat PAR_2F240S. Tc-YPGKF-NH_2 failed to have any effect on wild-type PAR-2 up to doses of 800μM. This was in complete contrast to the PAR_2F240S receptor that showed a robust response at concentrations of 100μM. This absolute contrast in receptor activation

between the wild-type and polymorphic receptor confirms that these two receptors are functionally very distinct, at least in the way they recognize agonists. Furthermore, the remarkable ability of PAR_2F240S receptor to be activated by peptides which have little resemblance to its' own peptide agonists, suggests that difficulties may arise in the development of PAR-2 antagonists. Furthermore, the possibility also arises that other small peptides found in vivo may activate this receptor, resulting in an inappropriate inflammatory response.

Accumulating evidence suggests that PAR-2 plays a key role in inflammation (19,21,22,38). PAR-2 is expressed at high levels in the gastrointestinal tract and has been demonstrated to regulate ion transport, gut motility and prostaglandin release (39-42). Interestingly, the bacterial protease Gingipain-R derived from *Porphyromonas gingivalis* activates PAR-2 (43), and this activation may provide one of the first inflammatory signals of pathogen invasion. Based on our current results, the presence of the PAR-2S polymorphism may be predictive of, or contribute to, certain inflammatory and intestinal disorders. In addition the PAR_2F240S receptor may have a deleterious effect in the lung, as PAR-2 has been reported to have a protective role in the lung by stimulating the release of the powerful bronchodilator prostaglandin E_2 (44). Thus, PAR_2F240S may be involved in diseases of the airways such as asthma. However, that said, little is currently known about the pathophysiological roles of PAR-2 and a better understanding will depend on the development of selective PAR-2 antagonists. Recently the potent inflammatory effects of PAR-2 activation have called into question the role of this receptor in disease and the possibility of PAR-2 antagonists as novel anti-inflammatory agents (45,46). To date only one true antagonist has been developed for PAR-1 (47) and as yet none are available for PAR-2. Considering the differential activation of PAR agonists found on this PAR-2 polymorphism we suspect that this receptor will need to be taken into account when designing PAR-2 antagonists. Whether this polymorphism is associated with a disease is yet to be determined, but considering the significant changes in agonist potencies and the relatively uncommon nature of the allele, it is feasible that this polymorphism may be a contributing factor of disease.

In conclusion, we have found a polymorphic variation of the human PAR-2 receptor at amino acid 240, where F (wild type) or S, can be found. This variation results in

significant differential receptor activation by trypsin and PAR agonists. Such variation may represent a genetic basis for interindividual differences in disease susceptibility, phenotype or response to therapeutic agents targeting PAR-2.

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CLAIMS

1. A variant protease activated receptor 2 (PAR-2) polypeptide or a fragment thereof which

(i) has reduced sensitivity to trypsin as compared with wild type PAR-2:

(ii) has increased sensitivity to trans-cinnamoyl-LIGRLO-NH_2 as compared with wild type PAR-2: and

(iii) is activated by TLIGRL-NH_2,

which polypeptide or fragment thereof comprises an extracellular loop 2 (ECL-2) having at least one amino acid difference from the corresponding ECL-2 amino acid sequence of the wild type polypeptide.

2. A polypeptide according to claim 1 wherein the wild type polypeptide is human PAR-2 having the amino acid sequence set out in SEQ ID No. 2.

3. A polypeptide according to claim 1 or 2 wherein the at least one amino acid difference includes a modification at residue 240 of the amino acid sequence set out in SEQ ID No.2, or its equivalent.

4. A polypeptide according to claim 3 comprising a substitution at residue 240 of the amino acid sequence set out in SEQ ID No. 2, or its equivalent.

5. A polypeptide according to claim 4 comprising a phenylalanine to serine substitution at residue 240 of the amino acid sequence set out in SEQ ID No.2, or its equivalent.

6. A PAR-2 polypeptide of fragment thereof which lacks a phenylalanine residue at position 240 of the amino acid sequence set out in SEQ ID No. 2, or it equivalent.

7. A nucleotide encoding a polypeptide according to any one of the preceding claims.

8. A host cell comprising a nucleotide according to claim 7 operably linked to a transcriptional control sequence capable of directing the expression of a polypeptide according to any one of claims 1 to 6 in the host cell.

9. A pair of oligonucleotide primers, each comprising at least 10 contiguous nucleotides of the polynucleotide of claim 7, which primers are suitable for use in a polymerase chain reaction for amplifying the ECL-2 region of a PAR-2 gene.

10. Use of the primers of claim 9 in a method of identifying an individual having a polymorphism in the ECL-2 region of one or both PAR-2 gene alleles.

11. A method of identifying a compound that modulates the activity of a polypeptide according to any one of claims 1 to 6 which method comprises contacting said polypeptide with a candidate compound and determining whether the candidate compound modulates the activity of said polypeptide.

12. A method of identifying a compound that selectively inhibits a polypeptide according to any one of claims 1 to 6 which method comprises:

(i) contacting said polypeptide with a candidate compound in the presence and absence of a PAR-2 agonist;

(ii) selecting those candidate compounds that inhibit or reduce the agonist-mediated activation of the polypeptide;

(iii) contacting a wild type PAR-2 polypeptide with a candidate compound selected in step (ii) in the presence and absence of a PAR-2 agonist; and

(iv) selecting those candidate compounds that do not significantly inhibit the agonist-mediated activation of the wild type PAR-2 polypeptide.

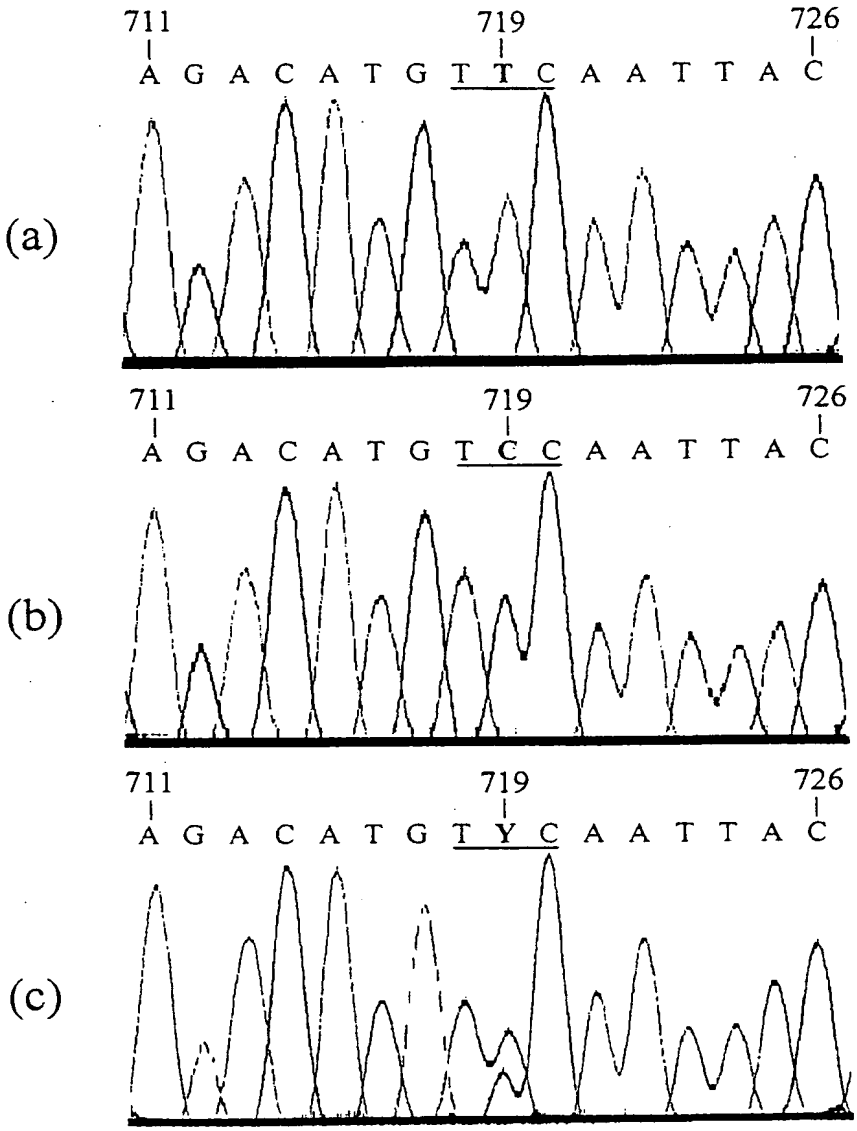
13. A compound identified by the method of claim 11 or 12.

14. A method of identifying a patient having an increased risk of inflammatory disorders which method comprises determining the presence or absence of a polypeptide according to any one of claims 1 to 6 or a polynucleotide according to claim 7 in a biological sample obtained from said patient.

15. A compound that modulates the activity of a polypeptide according to any one of claims 1 to 6 for use in a method of treating a condition characterised by inflammation.

16. A compound according to claim 15 that inhibits the activity of a polypeptide according to any one of claims 1 to 6.

Figure 1


711
719
726
A G A C A T G T T C A A T T A C
(a)
711
719
726
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(b)
711
719
726
A G A C A T G T Y C A A T T A C
(c)

Figure 2

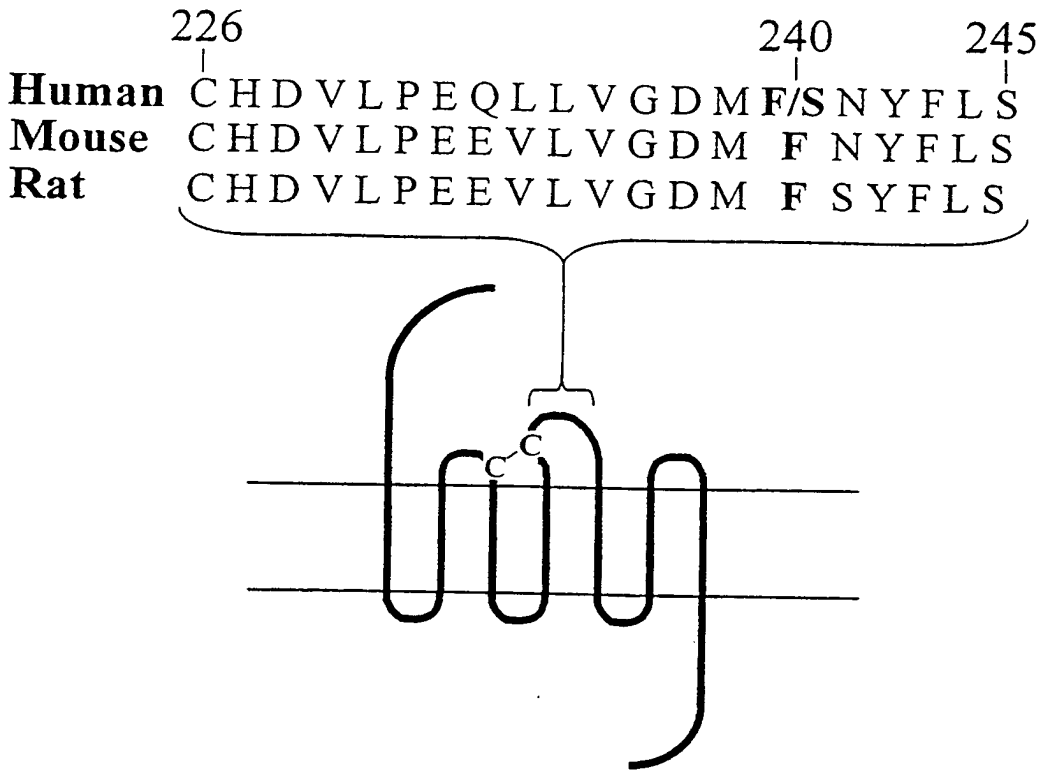


Figure 3

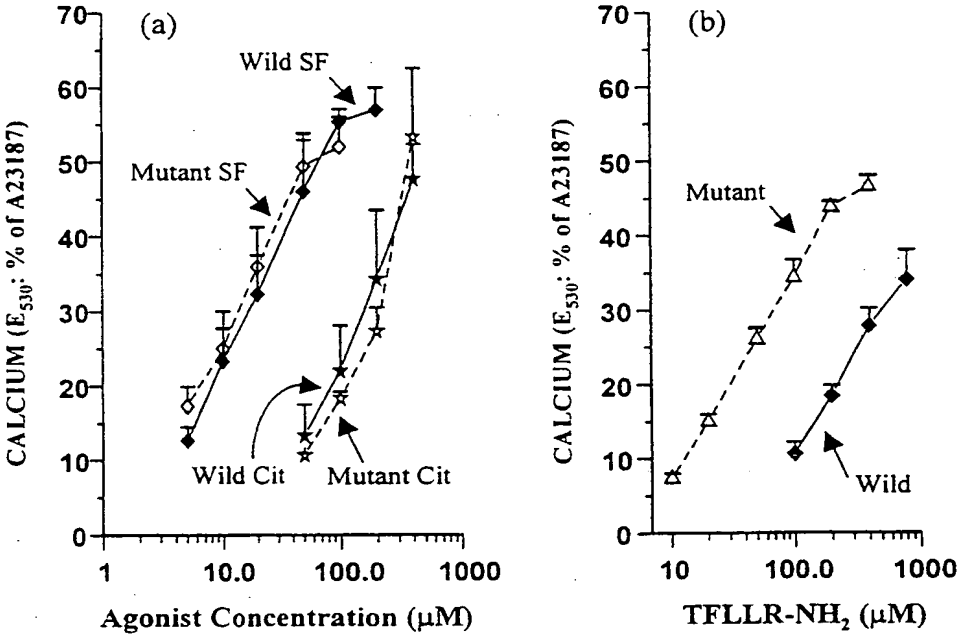


Figure 4

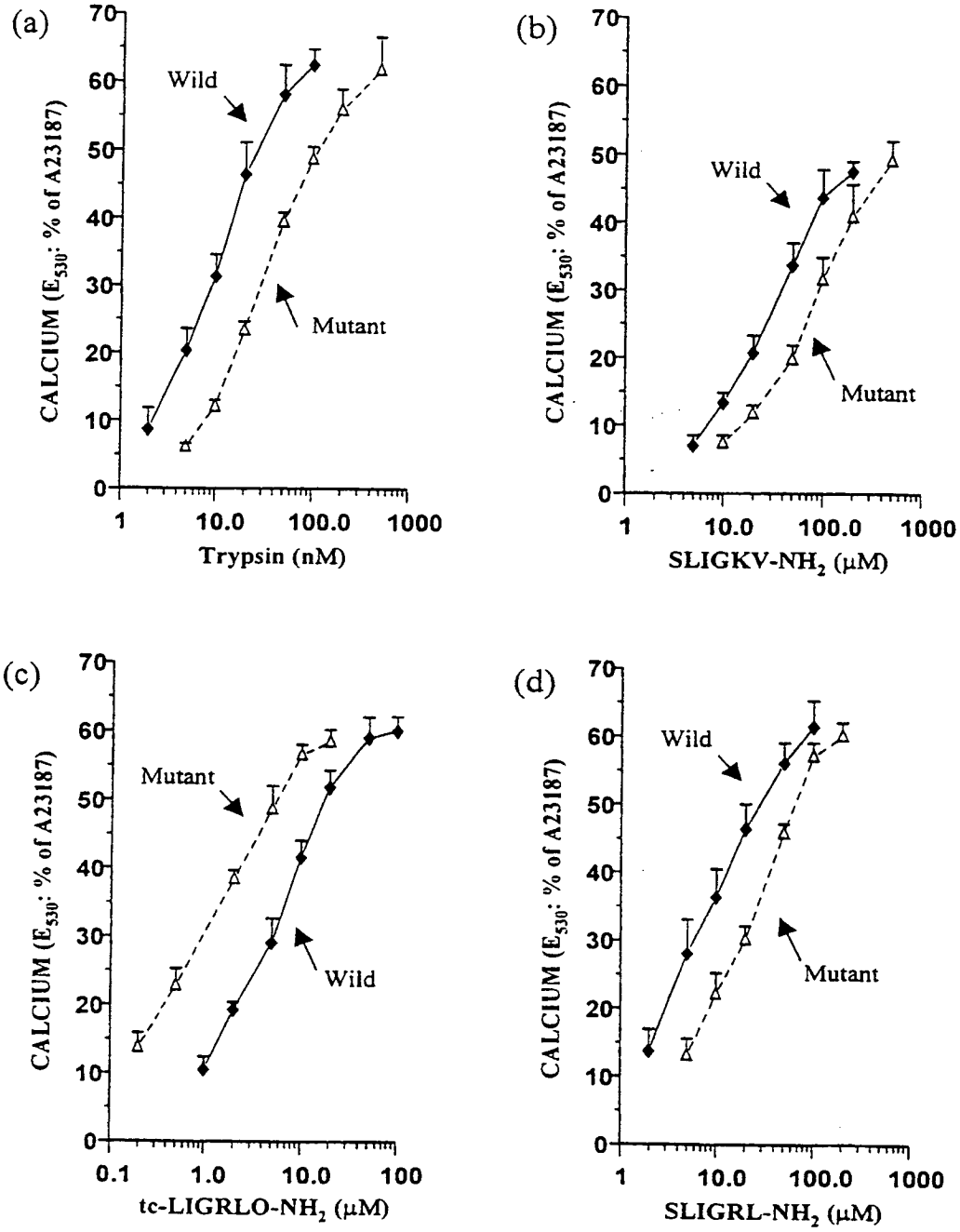
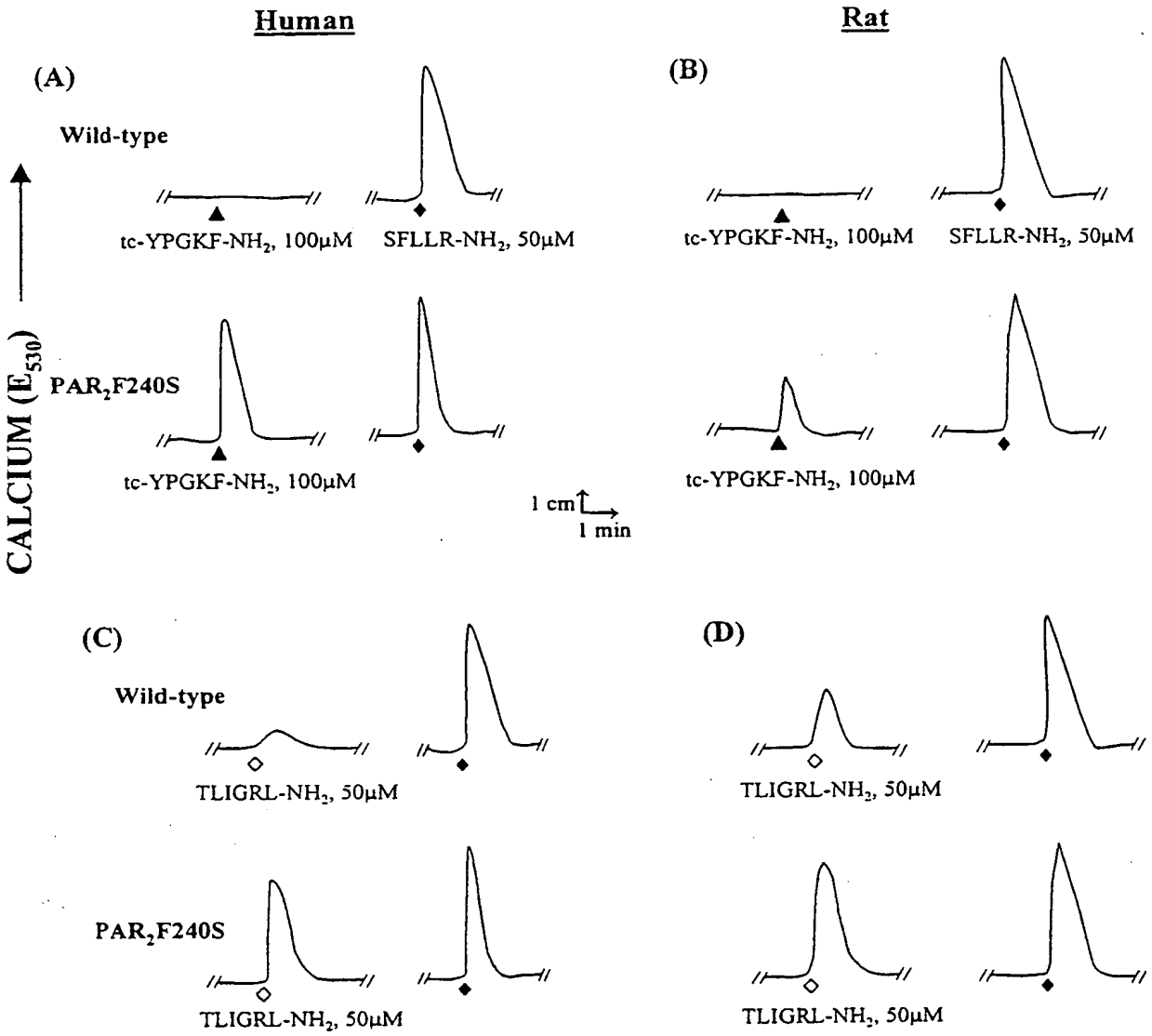


Figure 5



SEQUENCE LISTING PART OF THE DESCRIPTION

SEQ ID NO 1 – human PAR-2 nucleotide sequence

VERSION U34038.1 GI:1041728

FEATURES Location/Qualifiers

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/organism="Homo sapiens"
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/tissue_type="kidney"

5'UTR 1..147

CDS 148..1341
/note="G-protein-coupled receptor"
/codon_start=1
/product="proteinase-activated receptor-2"
/protein_id="AAB47871.1"
/db_xref="GI:1041729"

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 121 gcgcccggcg tcggggcttc caggaggatg cggagcccca gcgcggcgtg gctgctgggg
 181 gccgccatcc tgctagcagc ctctctctcc tgcagtggca ccatccaagg aaccaataga
 241 tcctctaaag gaagaagcct tattggtaag gttgatggca catcccacgt cactggaaaa
 301 ggagttacag ttgaaacagt cttttctgtg gatgagtttt ctgcatctgt cctcactgga
 361 aaactgacca cggtcttcct tccaattgtc tacacaattg tgtttgtggt gggtttgcca
 421 agtaacggca tggccctgtg ggtctttctt ttccgaacta agaagaagca ccctgctgtg
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 781 gtgaagcaga ccatcttcat tcctgccctg aacatcacga cctgtcatga tgttttgcct
 841 gagcagctct tggtgggaga catgttcaat tacttcctct ctctggccat tggggtcttt
 901 ctgttcccag ccttcctcac agcctctgcc tatgtgctga tgatcagaat gctgcgatct
 961 tctgccatgg atgaaaactc agagaagaaa aggaagaggg ccatcaaact cattgtcact
1021 gtcctggcca tgtacctgat ctgcttcact cctagtaacc ttctgcttgt ggtgcattat
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1201 agggatcatg caaagaacgc tctcctttgc cgaagtgtcc gcactgtaaa gcagatgcaa
1261 gtatccctca cctcaaagaa acactccagg aaatccagct cttactcttc aagttcaacc
1321 actgttaaga cctcctattg agttttccag gtcctcagat gggaattgca cagtaggatg
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SEQ ID NO 2 – human PAR-2 amino acid sequence

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121 DLLSVIWFPL KIAYHIHGNN WIYGEALCNV LIGFFYGNMY CSILFMTCLS VQRYWVIVNP
181 MGHSRKKANI AIGISLAIWL LILLVTIPLY VVKQTIFIPA LNITTCHDVL PEQLLVGDMF
241 NYFLSLAIGV FLFPAFLTAS AYVLMIRMLR SSAMDENSEK KRKRAIKLIV TVLAMYLICF
301 TPSNLLLVVH YFLIKSQGQS HVYALYIVAL CLSTLNSCID PFVYYFVSHD FRDHAKNALL
361 CRSVRTVKQM QVSLTSKKHS RKSSSYSSSS TTVKTSY
```

INTERNATIONAL SEARCH REPORT

Inte .onal Application No
PCT/GB 00/01455

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 C07K14/705 C12N5/10 C12Q1/68 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, WPI Data, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BOHM STEPHAN K ET AL: "Molecular cloning, expression and potential functions of the human proteinase-activated receptor-2." BIOCHEMICAL JOURNAL, vol. 314, no. 3, 1996, pages 1009-1016, XP000939053 ISSN: 0264-6021	9,13
Y	the whole document	10,11

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 September 2000	20/09/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Van der Schaal, C

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INTERNATIONAL SEARCH REPORT

Inte. onal Application No
PCT/GB 00/01455

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	COMPTON STEVEN J ET AL: "Molecular cloning of a protease-activated receptor-2 subtype." FASEB JOURNAL, vol. 13, no. 4 PART 1, 12 March 1999 (1999-03-12), page A325 XP002146868 Annual Meeting of the Professional Research Scientists for Experimental Biology 99;Washington, D.C., USA; April 17-21, 1999 ISSN: 0892-6638 abstract	10
X	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1997 HOLLENBERG MORLEY D ET AL: "Proteinase-activated receptors: Structural requirements for activity, receptor cross-reactivity, and receptor selectivity of receptor-activating peptides." Database accession no. PREV199799795932 XP002146869 abstract & CANADIAN JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY, vol. 75, no. 7, 1997, pages 832-841, ISSN: 0008-4212	13
X	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; December 1998 (1998-12) SAIFEDDINE MAHMOUD ET AL: "Endothelium-dependent contractile actions of proteinase-activated receptor-2-activating peptides in human umbilical vein: Release of a contracting factor via a novel receptor." Database accession no. PREV199900067484 XP002146870 abstract & BRITISH JOURNAL OF PHARMACOLOGY, vol. 125, no. 7, December 1998 (1998-12), pages 1445-1454, ISSN: 0007-1188	13
X	US 5 874 400 A (SUNDELIN JOHAN ET AL) 23 February 1999 (1999-02-23) cited in the application the whole document	13

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/01455

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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